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[^{3}H]Dipyridamole binding to nucleoside transporters from guinea-pig and rat lung

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Membranes from guinea-pig lung exhibited high-affinity binding of [^{3}H]dipyridamole, a potent inhibitor of nucleoside transport. Binding (apparent K_D 2 nM) was inhibited by the nucleoside-transport inhibitors nitrobenzylthioinosine (NBMPR), dilazep and lidoflazine and by the transported nucleosides uridine and adenosine. In contrast, there was no detectable high-affinity binding of [^{3}H]dipyridamole to lung membranes from the rat, a species whose nucleoside transporters exhibit a low sensitivity to dipyridamole inhibition. $B_{max.}$ values for high-affinity binding of [^{3}H]dipyridamole and [^{3}H]NBMPR to guinea-pig membranes were similar, suggesting that these structurally unrelated ligands bind to the NBMPR-sensitive nucleoside transporter with the same stoichiometry.

INTRODUCTION

Dipyridamole and the *S*-substituted 6-thiopurine ribonucleoside nitrobenzylthioinosine (NBMPR) are potent, structurally unrelated, inhibitors of facilitated-diffusion nucleoside transport in a wide variety of cell types and tissues (Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1981; Young & Jarvis, 1983). Both compounds have been used extensively in biochemical, physiological and pharmacological studies of nucleoside-transporter function. Clinically, dipyridamole has anti-thrombotic activity and a well-established action as a coronary vasodilator (Berne *et al.*, 1983). Nucleoside-transport inhibition by NBMPR is associated with high-affinity binding of ligand to cell-membrane sites (Cass *et al.*, 1974). Dissociation constants for specifically bound NBMPR at these sites in erythrocytes and other cell types are in the range 10^{-9}–10^{-10} M. In erythrocytes, it has been established that NBMPR binds to the outward face of the transporter (Jarvis *et al.*, 1982). Exposure of site-bound [^{3}H]NBMPR to high-intensity u.v. light results in specific covalent radiolabelling of membrane protein, identifying the human erythrocyte nucleoside transporter as a band 4.5 polypeptide with an apparent M_r on SDS/polyacrylamide gels of 45000–65000 (Wu *et al.*, 1983). NBMPR-sensitive nucleoside transporters in other cell types have been shown to have similar M_r to the erythrocyte system (see, for example, Kwan & Jarvis, 1984; Shi *et al.*, 1984; Young *et al.*, 1984). NBMPR-insensitive nucleoside transporters are also present in some cells (Belt, 1983; Belt & Noel, 1985).

Unlike NBMPR, the molecular mechanism by which dipyridamole and other vasodilators such as dilazep inhibit nucleoside-transporter function is poorly understood. It has been established that dipyridamole and dilazep compete with NBMPR in equilibrium-binding experiments in erythrocytes and cultured cells (Paterson, 1979; Paterson *et al.*, 1980; Jarvis *et al.*, 1983; Koren *et al.*, 1983). However, both agents inhibit NBMPR dissociation from membranes (Jarvis *et al.*, 1983; Koren *et al.*, 1983), raising the possibility that NBMPR and vasodilators interact with separate sites. Dipyridamole inhibition of [^{3}H]NBMPR binding to brain membranes is complex and species-dependent (Hammond & Clanachan, 1985). Recently, high-specific-radioactivity [^{3}H]dipyridamole has become commercially available (Marangos *et al.*, 1985). Using this ^{3}H-labelled ligand, Marangos *et al.* (1985) reported the presence of high-affinity binding sites for dipyridamole in guinea-pig brain membranes. The apparent K_D for dipyridamole binding (3.5 nM) was higher than for NBMPR (0.15 nM). Corresponding $B_{max.}$ values were 320 fmol/mg of protein for dipyridamole and 100 fmol/mg of protein for NBMPR, a ratio of 3.2:1. [^{3}H]Dipyridamole binding was inhibited by NBMPR. In the present paper we describe some of the characteristics of [^{3}H]dipyridamole binding to guinea-pig and rat lung membranes. Lung membranes were chosen for the present study because of their high density of NBMPR binding sites and because of the physiological role of the lung in removing circulating adenosine, the endogenous nucleoside whose action is potentiated by dipyridamole. Our results suggest that NBMPR and dipyridamole bind to the NBMPR-sensitive nucleoside transporter with the same stoichiometry.

MATERIALS AND METHODS

Materials

[^{3}H]Dipyridamole (sp. radioactivity 110 Ci/mmol, 96% radiochemically pure) and [^{3}H]NBMPR (sp. radioactivity 37 Ci/mmol, > 98% radiochemically pure) were purchased from Moravek Biochemicals, Brea, CA, U.S.A. Calibrated n-[^{3}H]hexadecane standard was obtained from Amersham International, Amersham, Bucks., U.K. Non-radioactive dipyridamole (Persantin injection) and NBMPR were purchased from Boehringer Ingelheim, Bracknell, Berks., U.K., and Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., respectively. Dilazep and lidoflazine were generously given by Roche

Abbreviations used: NBMPR, nitrobenzylthioinosine; $B_{max.}$, maximal binding; IC_{50}, concentration causing 50% inhibition.

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Pharmaceuticals and Professor A. R. P. Paterson, University of Alberta Cancer Research Unit, respectively.

Tissue and membrane preparation

Male Sprague–Dawley rats (400–450 g) and Dunkin–Hartley guinea pigs (700–800 g) were anaesthetized with diethyl ether and the lungs perfused *in situ* with heparinized saline to remove trapped erythrocytes. Lung tissue was then removed from the animals, washed twice in ice-cold saline and homogenized in 25 vol. (w/v) of ice-cold 50 mM-Tris/HCl, pH 7.4 at 22 °C, with a Brinkman Polytron PT-10 instrument (setting 5, 6 s). Samples were centrifuged at 45000 *g* for 10 min and the pellets washed three times in 25 vol. of Tris/HCl before resuspension in the same volume of buffer. This crude total membrane preparation was used without further purification. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Dipyridamole and NBMPR binding

Rat and guinea-pig lung membranes in 50 mM-Tris/HCl (0.05–0.08 mg of protein) were incubated with [^{3}H]dipyridamole (final equilibrium concentrations 0.05–30 nM) in a total volume of 1 ml at 20 °C in the absence and in the presence of 20 μM non-radioactive NBMPR or 50 μM-dipyridamole as competing ligands. Incubations (typically 30 min) were terminated on glass-fibre filters (Whatman GF/B, which were washed with ice-cold buffer before sample filtration). The filters were washed four times with 3 ml aliquots of ice-cold buffer, the whole procedure being completed in 15 s. The filters were dried, added to 10 ml of scintillation fluid and shaken at room temperature for 2 h before counting radioactivity. [^{3}H]Dipyridamole binding to membranes was expressed per mg of protein. Equilibrium concentrations of unbound [^{3}H]ligand were estimated from the differences between total [^{3}H]dipyridamole concentrations and the amounts of ligand bound to membranes and filters. Control time-course experiments confirmed that equilibrium was reached for all radioligand concentrations during the 30 min incubation period. $B_{max.}$ values for [^{3}H]NBMPR binding were determined at 5 nM-radioactive ligand ($\pm$20 μM non-radioactive NBMPR) as described previously (Shi *et al.*, 1984).

We noticed that [^{3}H]dipyridamole, in contrast with NBMPR, had a tendency to stick to the disposable polypropylene tubes and pipette tips used in the binding experiments. For this reason, [^{3}H]dipyridamole concentrations in the concentration-dependence experiments were determined directly by counting for radioactivity aliquots (0.1 ml) from each incubation immediately before filtration. ^{3}H radioactivity present in these aliquots (including those adsorbed by the pipette tips used in the transfer) was converted first to d.p.m. by using n-[^{3}H]hexadecane as internal standard and then to ligand concentration (nM) by using the [^{3}H]dipyridamole specific radioactivity value supplied by Moravek Biochemicals. A similar procedure was used to convert membrane-bound c.p.m. into pmol. For the sake of consistency, values for membrane-bound [^{3}H]NBMPR were determined in the same way.

RESULTS AND DISCUSSION

We have previously established that guinea-pig and rat lung membranes possess a high density of nucleoside transporters, as judged by assays of high-affinity [^{3}H]NBMPR binding activity (Shi *et al.*, 1984). $B_{max.}$ values for [^{3}H]NBMPR binding were determined to be in the region of 20 and 10 pmol/mg of protein for guinea-pig and rat lung membranes respectively, with apparent K_D values of 0.2–0.4 nM. In the present study we investigated the binding of [^{3}H]dipyridamole to these two lung membrane preparations.

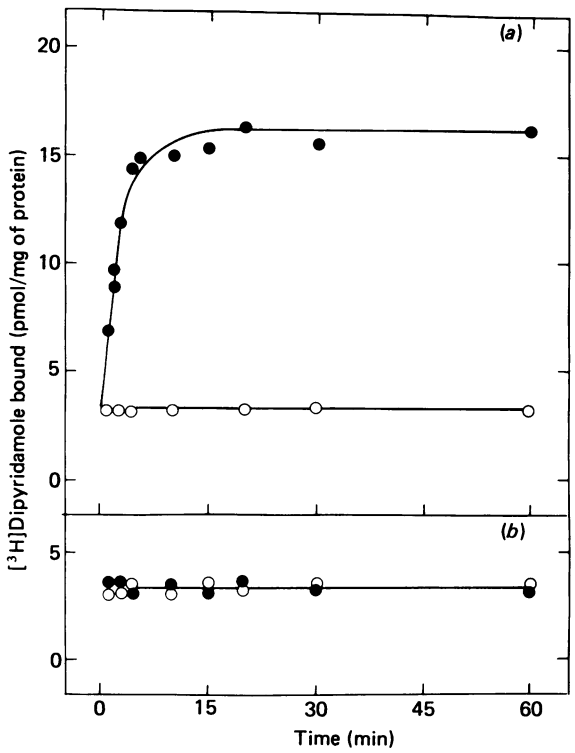

Fig. 1. Time-course of [^{3}H]dipyridamole binding to guinea-pig (*a*) and rat lung membranes (*b*)

Binding of 5 nM-[^{3}H]dipyridamole was measured at room temperature in the absence (●) and in the presence (○) of 20 μM-NBMPR as competing non-radioactive ligand. Values are means of duplicate determinations.

A representative time course for [^{3}H]dipyridamole binding (initial concentration 5 nM) to guinea-pig lung membranes, measured both in the absence and in the presence of 20 μM-NBMPR as competing non-radioactive ligand, is shown in Fig. 1(*a*). The results demonstrate the presence of a large component of NBMPR-sensitive binding (12.8 pmol/mg of protein). The half-time for [^{3}H]dipyridamole binding to these sites at 20 °C was approx. 2 min, full equilibration occuring within 15 min. [^{3}H]Dipyridamole binding in the presence of NBMPR, which includes adsorption of radioactive ligand by the filters as well as non-specific (NBMPR-insensitive) binding to membranes, was not time-dependent and accounted for 19.8% of the total binding at equilibrium. In control experiments we found that [^{3}H]ligand binding to glass-fibre filters was unaffected by NBMPR.

In marked contrast with guinea-pig membranes, there was no detectable NBMPR-sensitive binding of [^{3}H]dipyridamole to membranes from rat lung (Fig. 1*b*). This result is in accord with previous studies suggesting that rat nucleoside transporters have a low affinity for dipyridamole (see, for example, Marangos *et al.*, 1984;

Hammond & Clanachan, 1984). Thus it has been determined that non-radioactive dipyridamole is a competitive inhibitor of [³H]NBMPR binding to guinea-pig lung membranes with an apparent K_i of 18 nM compared with a value of 8.5 μM for rat membranes, a difference of 470-fold (Shi & Young, 1986). We would therefore not expect to detect binding of [³H]dipyridamole to rat lung nucleoside transporters, membranes from this species effectively representing a negative control for transporter-associated ligand binding.

Fig. 2(*a*) presents the concentration-dependence of [³H]dipyridamole binding to guinea-pig lung membranes, measured at equilibrium (30 min incubation) in the absence and in the presence of 20 μM-NBMPR. Binding activity is plotted against the calculated free concentrations of unbound ligand in the medium. NBMPR-sensitive binding of [³H]dipyridamole to the membranes was saturable, with a Hill coefficient of 1.12. Scatchard analysis of the data gave an apparent K_D value of 1.9 nM. This affinity constant compares with an apparent K_D of 3.5 nM for [³H]dipyridamole binding to guinea-pig brain membranes (Marangos *et al.*, 1985) and an apparent K_i value of 18 nM determined for dipyridamole inhibition of equilibrium [³H]NBMPR binding to guinea-pig lung membranes (see above). The latter value was determined by using non-radioactive dipyridamole and therefore takes no account of ligand depletion by membranes or adsorption of ligand by incubation tubes etc. The $B_{max.}$ value for NBMPR-sensitive [³H]dipyridamole binding in the experiment shown in Fig. 2(*a*) was 25.5 pmol/mg of protein, compared with a $B_{max.}$ of 17.1 pmol/mg of protein for [³H]NBMPR binding to the same membrane preparation. Mean $B_{max.}$ values (±S.E.M.) for four different membrane preparations were 24.8±4.0 and 19.9±1.5 pmol/mg of protein for [³H]dipyridamole and [³H]NBMPR respectively, 70-fold greater than [³H]dipyridamole binding to guinea-pig brain membranes (Marangos *et al.*, 1985). As also shown in Fig. 2(*a*), high-affinity binding of [³H]dipyridamole to guinea-pig lung membranes was abolished in the presence of excess non-radioactive dipyridamole (50 μM). Non-saturable [³H]ligand binding in the presence of dipyridamole was less than with NBMPR, indicating the occurrence of low-affinity radioligand binding (to membranes or filters) unrelated to NBMPR-sensitive nucleoside transport. Data from a comparable experiment using membranes prepared from rat lung are shown in Fig. 2(*b*). As expected from the results presented in Fig. 1(*b*), no NBMPR-sensitive binding of [³H]dipyridamole was detected. Thus [³H]dipyridamole binding was non-saturable and co-plotted with radioligand binding measured in the presence of 20 μM-NBMPR. As in guinea-pig membranes, [³H]ligand binding in the presence of 50 μM-dipyridamole was lower, the difference amounting to the equivalent of 2.5 pmol/mg of protein (10 nM-[³H]ligand) in both species. The [³H]NBMPR binding capacity of the rat membranes used in this experiment was 8.2 pmol/mg of protein.

In a final series of experiments we tested the transported nucleosides adenosine and uridine and the vasodilators dilazep and lidoflazine for their ability to inhibit high-affinity (NBMPR-sensitive) binding of [³H]dipyridamole to guinea-pig lung membranes. As shown in Table 1, both nucleosides competed with the [³H]ligand for binding to the high-affinity sites. Adenosine was more effective than uridine (IC_{50} 0.95 and 7.2 mM respectively at 5 nM-[³H]dipyridamole), in agreement with their relative affinities for the nucleoside transporter in different cell types (see below). Similarly, dilazep and lidoflazine inhibited high-affinity (NBMPR-sensitive) binding of [³H]dipyridamole to guinea-pig

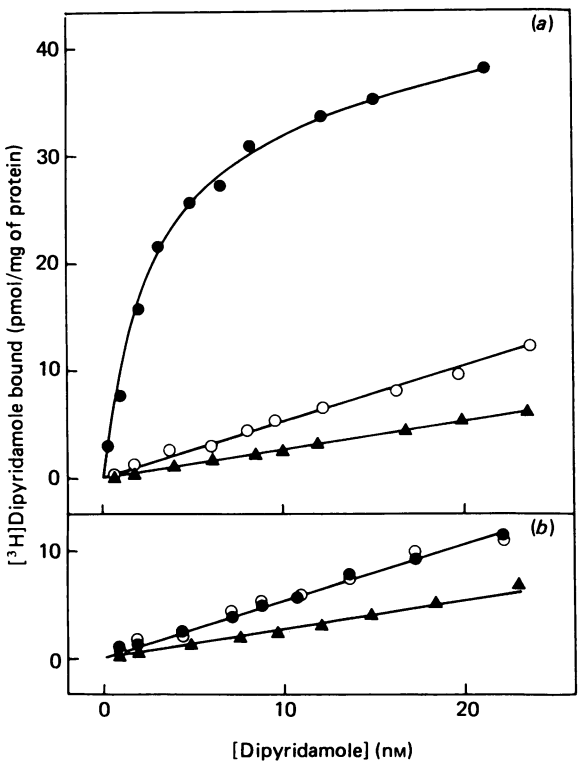

Fig. 2. Concentration-dependence of [³H]dipyridamole binding to guinea-pig (*a*) and rat lung membranes (*b*)

Binding of [³H]dipyridamole was measured at room temperature (30 min incubation) in the absence (●) and in the presence of 20 μM-NBMPR (○) or 50 μM-non-radioactive dipyridamole (▲). Values are means of triplicate determinations. Standard errors (not shown) were typically less than 10% of mean values.

Table 1. Effects of nucleosides on high-affinity binding of [³H]dipyridamole to guinea-pig lung membranes

Binding of [³H]dipyridamole (5 nM) was measured at room temperature (30 min incubation) in the absence and in the presence of various concentrations of competing nucleoside and corrected for non-specific radioligand binding measured in the presence of 20 μM-NBMPR (see Fig. 2). Adenosine and uridine (10 mM) had no measurable effect on NBMPR-insensitive binding of 5 nM-[³H]dipyridamole (98.0±1.9 and 99.5±1.5% of control NBMPR-insensitive binding respectively). Values are means±S.E.M. of triplicate determinations.

Nucleoside	Concn. (mM)	Binding (% of control)
Adenosine	0.1	73.5±5.2
	1.0	53.0±5.6
	10.0	24.7±1.4
Uridine	0.1	83.7±4.4
	1.0	65.8±6.3
	10.0	48.6±4.5

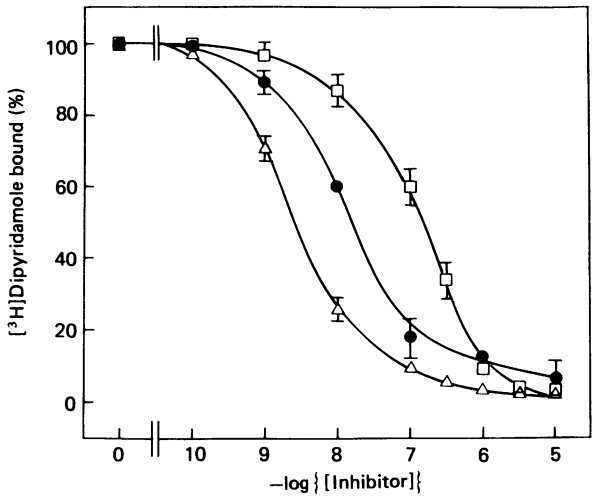

Fig. 3. Effects of dilazep, lidoflazine and NBMPR on high-affinity binding of [^{3}H]dipyridamole to guinea-pig lung membranes

Binding of [^{3}H]dipyridamole (5 nM) was measured at room temperature (30 min incubation) in the absence and in the presence of various concentrations of dilazep (●), lidoflazine (□) and NBMPR (△) and corrected for non-specific radioligand binding measured in the presence of 20 μM-NBMPR. Dilazep and lidoflazine at a concentration of 10 μM had no significant effect on NBMPR-insensitive binding of 5 nM-[^{3}H]dipyridamole (97.2±2.1 and 94.5±2.2% of control NBMPR-insensitive binding respectively). Values are means ±S.E.M. of triplicate determinations.

membranes (Fig. 3). IC_{50} values for this inhibition were 16 and 140 nM respectively (5 nM-[^{3}H]dipyridamole). For comparison, the estimated IC_{50} value for NBMPR inhibition of [^{3}H]dipyridamole binding was 3.2 nM (see also Fig. 3). Within experimental error, dilazep, lidoflazine and NBMPR displaced maximally the same amount of radioligand. Pseudo Hill coefficients for this inhibition were 0.82, 0.86 and 0.88 respectively. None of these compounds had significant effects on NBMPR-insensitive [^{3}H]dipyridamole binding (see the legends to Table 1 and Fig. 3), additional evidence that this component of radioligand binding is not related to nucleoside transport.

IC_{50} concentrations estimated from the data presented in Table 1 and Fig. 3 were used to determine apparent K_i values from the relationship:

$$K_i = IC_{50}/[1+([L]/K_D)]$$

where [L] is the radioligand concentration and K_D its binding affinity. Calculated inhibitor constants were: adensoine, 0.26 mM; uridine, 1.93 mM; dilazep, 4.4 nM; lidoflazine, 39 nM; NBMPR, 0.9 nM. Although these K_i values, based on a single [^{3}H]dipyridamole concentration, can only be regarded as approximate, they were found to correspond well to equivalent K_i values determined for inhibition of high-affinity [^{3}H]NBMPR binding to guinea-pig lung membranes: adenosine, 0.28 mM; uridine, 2.0 mM; dilazep, 2.0 nM; lidoflazine, 110 nM (Shi *et al.*, 1984; M. M. Shi & J. D. Young, unpublished work). The apparent K_i for NBMPR inhibition of [^{3}H]dipyridamole binding (0.9 nM) also agrees well with the apparent K_D value for [^{3}H]NBMPR binding to these membranes (0.4 nM) (Shi *et al.*, 1984). With respect to the nucleoside permeants adenosine and uridine, corresponding apparent K_i values for inhibition of [^{3}H]NBMPR binding to erythrocyte membranes are 0.1 mM for adenosine (Jarvis *et al.*, 1983) and 1.0–1.3 mM for uridine (Jarvis *et al.*, 1982).

Thus the present series of experiments demonstrate that guinea-pig lung membranes possess high-affinity binding sites for dipyridamole, this binding representing a specific interaction with the NBMPR-sensitive nucleoside transporter. Our results further suggest that dipyridamole and NBMPR bind to the transporter with the same stoichiometry. In contrast, the study of [^{3}H]dipyridamole binding to guinea-pig brain membranes by Marangos *et al.* (1985) reported a 3.2-fold higher $B_{max.}$ for dipyridamole than for NBMPR. However, unlike the present investigation, that of Marangos *et al.* (1985) used non-radioactive dipyridamole to correct for non-specific binding. Our experiments suggest that this might give an erroneously high estimate of high-affinity binding of dipyridamole, particularly in tissues such as the central nervous system with a low density of binding sites. Physiological explanations for the discrepancy between dipyridamole and NBMPR binding in brain membranes include the possibility of high-affinity dipyridamole binding to NBMPR-insensitive nucleoside transporters of the type described by Belt (1983) and Belt & Noel (1985). Experiments in our laboratory have identified mixed NBMPR-sensitive and NBMPR-insensitive uridine transport in primary cultures of differentiated mouse astrocytes (Wu *et al.*, 1986). Both components of transport were inhibited by dipyridamole.

We conclude that [^{3}H]dipyridamole has considerable potential as an alternative probe to study molecular aspects of nucleoside-transporter function.

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